FORM PTO-1390 (Modified) (REV 11-98) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: **RDID0063US**

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR): **09/647586**

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP99/02361	**7 April 1999 (07.04.99)**	**7 April 1998 (07.04.98)**

TITLE OF INVENTION: **NEW COMPOUNDS FOR DNA-TRANSFECTION**

APPLICANT(S) FOR DO/EO/US: **KITAS, Eric Argirios and SCHLAEGER, Ernst-Jurgen**

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ A copy of the International Search Report (PCT/ISA/210).
8. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)). (unexecuted)
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 13 to 20 below concern document(s) or information included:

13. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment. (to follow)
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☒ Certificate of Mailing by Express Mail
20. ☒ Other items or information:

General Appointment of Representative for U.S. Patent and Trademark Application Matters; and Return postcard.

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/647586	PCT/EP99/02361	RDID0063US

21. The following fees are submitted:.

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

		CALCULATIONS PTO USE ONLY
☐	Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2) paid to USPTO and International Search Report not prepared by the EPO or JPO	**$970.00**
☒	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but Internation Search Report prepared by the EPO or JPO	**$840.00**
☐	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO	**$690.00**
☐	International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4)	**$670.00**
☐	International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4)	**$96.00**

ENTER APPROPRIATE BASIC FEE AMOUNT = **$840.00**

Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)). **$0.00**

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	- 20 =	0	x $18.00	**$0.00**
Independent claims	- 3 =	0	x $78.00	**$0.00**
Multiple Dependent Claims **(check if applicable)**.			☐	**$0.00**

TOTAL OF ABOVE CALCULATIONS = **$840.00**

Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) **(check if applicable)**. ☐ **$0.00**

SUBTOTAL = **$840.00**

Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). + **$0.00**

TOTAL NATIONAL FEE = **$840.00**

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☐ **$0.00**

TOTAL FEES ENCLOSED = **$840.00**

Amount to be: refunded	$
charged	$

☐ A check in the amount of to cover the above fees is enclosed.

☒ Please charge my Deposit Account No. **50-0877** in the amount of **$840.00** to cover the above fees.
A duplicate copy of this sheet is enclosed.

☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **50-0877.** A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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SIGNATURE

Kenneth J. Waite
NAME

45,189
REGISTRATION NUMBER

2 October 2000
DATE

EXPRESS MAIL: EK939496545US

Docket No. RDID0063 US

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: KITAS et al.

Application No.: 09/647,586

Filed: 02 October 2000

For: *New Compounds for DNA* Transfection

Assistant Commissioner for Patents
Box PCT
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Sir:

Please enter the following amendments prior to examination of the above-referenced application:

In the specification

Please amend the specification as follows:

On page 1 line 1 please add Background and Summary of the Invention

On page 2 line 30 please add Detailed Description of the Invention

In the claims

Please cancel claims 10-12, 16, 18, 19, and 22-24 without prejudice or disclaimer of the subject matter claimed therein.

Please amend claims 3-7, 13-15, and 17, and 20-21 as follows:

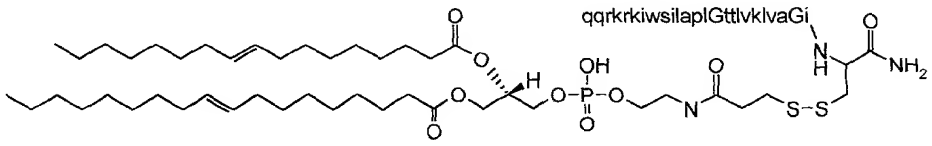
3. (Amended) The compounds of [claims 1 or 2] claim 1 wherein R^1 and R^2 are independently selected from lauroyl, palmitoyl, stearoyl and oleoyl.

4. (Amended) The compounds of [claims 1 - 3] claim 1 wherein X is -S-S-.

In claim 5, please change "claims 1-4" to --claim 1--.

In claim 6, please change "claim 5" to --claim 1--.

7. (Amended) The compound [according to any of claims 1 - 6] of claim 1, which is



(IV)

13. (Amended) The composition of [claims 10-12] claim 36 wherein the components are in the form of an aqueous or organic solution, an aqueous or organic dispersion, or a liposome or a micelle.

14. (Amended) Use of a composition as defined [in any one of claims 10-13] in claim 36 for transfecting a eukaryotic or prokaryotic cell in vivo or in vitro with an anionic macromolecule.

15. (Amended) Use of a composition as defined [in any one of claims 10-13] in claim 36 for transfecting a eukaryotic or prokaryotic cell in vivo or in vitro with a polynucleotide.

17. (Amended) Use of a compound as defined [in any one of claims 1-9] in claim 1 for transfecting a cell in vivo or in vitro with a polynucleotide.

20. (Amended) Use of a compound as defined [in any one of claims 1-9] in claim 1 for introducing in vivo or in vitro a biologically active molecule into cells.

21. (Amended) Use of a composition as defined [in any one of claims 10-13] in claim 36 for introducing in vivo or in vitro a biologically active molecule into cells.

Please add new claims 25-54 as follows:

25. Use of a compound as defined in claim 1 for transfecting a eukaryotic or prokaryotic cell in vivo or in vitro with an anionic macromlecule.

26. The compounds of claim 2 wherein R^1 and R^2 are independently selected from lauroyl, palmitoyl, stearoyl and oleoyl.

27. The compounds of claim 2 wherein X is -S-S-.

28. The compounds of claim 3 wherein X is -S-S.

29. The compounds of claim 2 wherein R^3 is Gln-Gln-Arg-Lys-Arg-Lys-Ile-Trp-Ser-Ile-Leu-Ala-Pro-Leu-Gly-Thr-Thr-Leu-Val-Lys-Leu-Val-Ala-Gly-Ile-NH-CH[$CONH_2$]-(CH_2)- with a reversed amide backbone or derivatives thereof consisting of at least 50 % D-amino acids.

30. The compounds of claim 3 wherein R^3 is Gln-Gln-Arg-Lys-Arg-Lys-Ile-Trp-Ser-Ile-Leu-Ala-Pro-Leu-Gly-Thr-Thr-Leu-Val-Lys-Leu-Val-Ala-Gly-Ile-NH-CH[$CONH_2$]-(CH_2)- with a reversed amide backbone or derivatives thereof consisting of at least 50 % D-amino acids.

31. The compounds of claim 4 wherein R^3 is Gln-Gln-Arg-Lys-Arg-Lys-Ile-Trp-Ser-Ile-Leu-Ala-Pro-Leu-Gly-Thr-Thr-Leu-Val-Lys-Leu-Val-Ala-Gly-Ile-NH-CH[$CONH_2$]-(CH_2)- with a reversed amide backbone or derivatives thereof consisting of at least 50 % D-amino acids.

32. The compounds of claim 2 wherein R^3 is D-Gln-D-Gln-D-Arg-D-Lys-D-Arg-D-Lys-D-Ile-D-Trp-D-Ser-D-Ile-D-Leu-D-Ala-D-Pro-D-Leu-Gly-D-Thr-D-Thr-D-Leu-D-Val-D-Lys-D-Leu-D-Val-D-Ala-Gly-D-Ile-NH-[$CONH_2$]-CH-(CH_2)-.

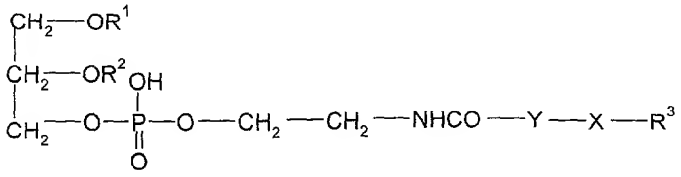
33. The compounds of claim 3 wherein R^3 is D-Gln-D-Gln-D-Arg-D-Lys-D-Arg-D-Lys-D-Ile-D-Trp-D-Ser-D-Ile-D-Leu-D-Ala-D-Pro-D-Leu-Gly-D-Thr-D-Thr-D-Leu-D-Val-D-Lys-D-Leu-D-Val-D-Ala-Gly-D-Ile-NH-[$CONH_2$]-CH-(CH_2)-.

34. The compounds of claim 4 wherein R^3 is D-Gln-D-Gln-D-Arg-D-Lys-D-Arg-D-Lys-D-Ile-D-Trp-D-Ser-D-Ile-D-Leu-D-Ala-D-Pro-D-Leu-Gly-D-Thr-D-Thr-D-Leu-D-Val-D-Lys-D-Leu-D-Val-D-Ala-Gly-D-Ile-NH-[$CONH_2$]-CH-(CH_2)-.

35. The compounds of claim 5 wherein R^3 is D-Gln-D-Gln-D-Arg-D-Lys-D-Arg-D-Lys-D-Ile-D-Trp-D-Ser-D-Ile-D-Leu-D-Ala-D-Pro-D-Leu-Gly-D-Thr-D-Thr-D-Leu-D-Val-D-Lys-D-Leu-D-Val-D-Ala-Gly-D-Ile-NH-[$CONH_2$]-CH-(CH_2).

36. A composition comprising at least at least one compound of formula:

(I)



wherein R^1 and R^2 are a hydrocarbyl moiety of a straight-chain or branched-chain, saturated or unsaturated aliphatic carboxylic acid or a phospholipid moiety, R^3 is a basic, membrane disturbing peptide with a reversed amide backbone, Y is C_{2-10} alkylene, X is -C(O)-NH- or -S-S- and salts thereof and a helper lipid.

37. The composition of claim 36 further comprising a short chain phospholipid.

38. The composition of claim 37 further comprising a cationic lipid.

39. The composition of claim 38 further comprising an additional transfection reagent.

40. The composition of claim 36 further comprising a cationic lipid.

41. The composition of claim 36 further comprising an anionic macromolecule.

42. The composition of claim 41 wherein the anionic macromolecule is a polynucleotide.

43. The composition of claim 41 further comprising a polycationic polymer.

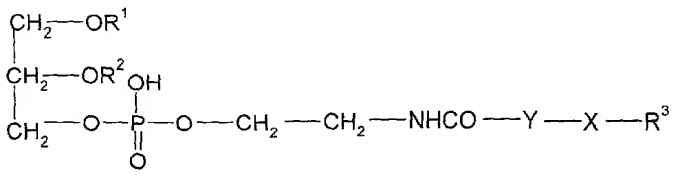
44. The composition of claim 43 wherein the polycationic polymer is polyethyleneimine.

45. The composition of claim 36 further comprising a polycationic polymer.

46. The composition of claim 45 wherein the polycationic polymer is polyethyleneimine.

47. A process for transfecting a cell in vivo or in vitro with an anionic macromolecule, comprising contacting a cell in vivo or in vitro with the anionic macromolecule in the presence of compounds of formula:

(I)

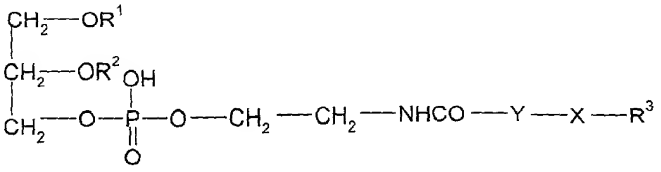


wherein R^1 and R^2 are a hydrocarbyl moiety of a straight-chain or branched-chain, saturated or unsaturated aliphatic carboxylic acid or a phospholipid moiety, R^3

is a basic, membrane disturbing peptide with a reversed amide backbone, Y is C_{2-10} alkylene, X is -C(O)-NH- or -S-S- and salts thereof.

48. A process for transfecting a cell in vivo or in vitro with an anionic macromolecule, comprising contacting a cell in vivo or in vitro with the anionic macromolecule in the presence of a composition comprising at least one compound of formula:

(I)



wherein R^1 and R^2 are a hydrocarbyl moiety of a straight-chain or branched-chain, saturated or unsaturated aliphatic carboxylic acid or a phospholipid moiety, R^3 is a basic, membrane disturbing peptide with a reversed amide backbone, Y is C_{2-10} alkylene, X is -C(O)-NH- or -S-S- and salts thereof and a helper lipid.

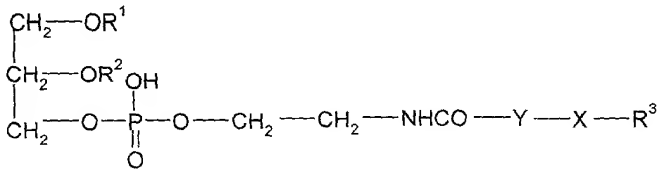
49. The process of claim 48, wherein the composition further comprises a short chain phospholipid.

50. The process of claim 49, wherein the composition further comprises a cationic lipid.

51. The process of claim 48, wherein the composition further comprises a cationic lipid.

52. A process for introducing a biologically active anionic molecule into a cell in vivo or in vitro with an anionic macromolecule, comprising contacting a cell in vivo or in vitro with the anionic macromolecule in the presence of a composition comprising at least one compound of formula:

(I)

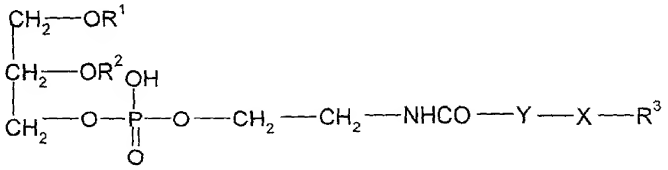


wherein R^1 and R^2 are a hydrocarbyl moiety of a straight-chain or branched-chain, saturated or unsaturated aliphatic carboxylic acid or a phospholipid moiety, R^3 is a

basic, membrane disturbing peptide with a reversed amide backbone, Y is C_{2-10} alkylene, X is -C(O)-NH- or -S-S- and salts thereof and a helper lipid.

53. A process for introducing in vivo or in vitro a biologically active anionic molecule into a cell, comprising contacting a cell in vivo or in vitro with the anionic macromolecule in the presence of a compound of formula:

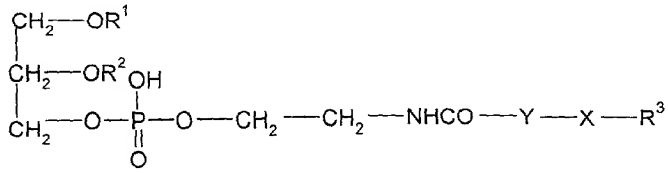
(I)



wherein R^1 and R^2 are a hydrocarbyl moiety of a straight-chain or branched-chain, saturated or unsaturated aliphatic carboxylic acid or a phospholipid moiety, R^3 is a basic, membrane disturbing peptide with a reversed amide backbone, Y is C_{2-10} alkylene, X is -C(O)-NH- or -S-S- and salts thereof.

54. A process for introducing in vivo or in vitro a biologically active anionic molecule into a cell, comprising contacting in vivo or in vitro a cell with the anionic macromolecule in the presence of a composition comprising at least one compound of formula:

(I)



wherein R^1 and R^2 are a hydrocarbyl moiety of a straight-chain or branched-chain, saturated or unsaturated aliphatic carboxylic acid or a phospholipid moiety, R^3 is a basic, membrane disturbing peptide with a reversed amide backbone, Y is C_{2-10} alkylene, X is -C(O)-NH- or -S-S- and salts thereof and a helper lipid.

REMARKS

The specification has been amended at page 1 line 1 and page 2 line 30 to add headings to the application. No new matter is believed to be added by virtue of the amendments to the specification.

Claim 16 was missing from the application as filed and therefore claim 16 has been cancelled. In addition, claims 10-12, 18, 19, and 22-24 have been cancelled without prejudice or disclaimer of the subject matter claimed therein. Claims 3-7, 13-15, 17, and 20-21 have been amended to remove their multiple dependencies. Claims 25-51 have been added to point out more specifically the subject matter of the present invention. Support for the new claims is found throughout the specification. No new matter is added by virtue of the amendments to the claims or of the new claims.

The claims as submitted herein are believed to be in condition for allowance, and allowance of the application is respectfully requested. In addition, it is requested that any fees due be charged to Deposit Account Number 50-0877 with reference to (RDID 0063 US).

Respectfully submitted,

Date: 22 Dec 00

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NEW COMPOUNDS FOR DNA-TRANSFECTION

The present invention is concerned with novel compounds especially useful for non-viral introduction of biologically active molecules such as DNA, RNA, peptides or proteins into eukaryotic cells.

Non-viral systems have been developed to carry DNA into cells, e.g., the transfection technique based on a cationic lipid, the dioleoyloxypropyl trimethylammonium (Felgner et al., Proc. Natl., Acad. Sci. USA, 1987, 84, 7413-7417) commercialized as Lipofectin™. Since the discovery of this transfection technique, many more cationic lipids have been synthesized and some are commercially available as transfecting reagent for laboratory use: DOGS (Transfectam™), DOSPA (Lipofectamine™), DOTAP (DOTAP™).

Transfection of cells with oligonucleotides such as DNA can be used, for example to express in a host cell or organism, a protein which is not normally expressed by that cell or organism. For example, a DNA molecule called a plasmid may be introduced into a cell that does not normally contain the gene (s) encoded by that plasmid in order to express a marker gene product in that cell, or to express a protein of interest such as a recombinant protein which is later harvested from such cells (See Sambrook, et al., Molecular Cloning: A Laboratory Manual, 2nd ed. (Cold Spring Harbor, 1989), ch. 1.). The transfection of oligonucleotides into cells can also be used therapeutically. For example, antisense oligonucleotides, once in the cell or cell nucleus, bind to target single-stranded nucleic acid molecules by Watson-Crick base pairing or to double stranded nucleic acids by Hoogsteen base pairing, and in doing so disrupt the function of the target by one of several mechanisms: by preventing the binding of factors required for normal transcription, splicing, or translation; by triggering the enzymatic degradation of mRNA by RNAse H, or by destroying the target via reactive groups attached directly to the antisense oligonucleotide. (See Zamecnic et al., Proc. Natl. Acad. Sci.-USA, 1978, 75, 280-284). Gene therapy or DNA based vaccination are other therapeutic applications.

Proteins and other macromolecules are transfected into cells for therapeutic and screening purposes. For example, immunization is enhanced by introducing an immunogenic protein into a cell, so that it is more efficiently processed and presented on the surface of the cells, thereby enhancing the immunogenic response. Negatively charged macromolecules which act

inside a cell are transported past the cell membrane into the cytoplasm where they exert their effect. Factors which enhance or hinder transcription of DNA can be used in a screening test to verify the transcription of a gene of interest. These transcription assays are very well known for use in screening compounds to determine their effect against a particular macromolecule, for example a cell receptor.

Cell-lytic antibacterial peptides that act by perturbing the barrier function of membranes are reviewed in Saberwal, et al., Biochim. Biophys. Acta, 1994, 1197, 109-131. Certain fatty acid-bearing basic peptides having antibacterial activity are disclosed in Vogler, et al., Helv. Chim. Acta, 1964, 47, 526-543. Poly(lysine-serine) random polymers for use as carriers to transport oligonucleotides into cells is disclosed in European Patent Publication No. EP-A-727 223.

European Patent Publication No. EP-A-784 984 and Legendre et al. (Bioconjugate Chem., 1997, 8, 57-63) describe conjugates of a lipid and a basic, membrane disturbing peptide that bind polynucleotides and anionic macromolecules can be used for transfection of cells. The peptide portion of the conjugate consists of natural amino acids linked by a natural amide binding.

Nevertheless, despite important progress in the formulation of non-viral gene delivery systems, there remains a need for more efficient techniques, since the transfection efficiency of synthetic systems is usually below that of viral vectors. Furthermore, still many problems arise in vivo and in vitro due in part to the poor stability of the non-viral systems in biological fluids and culture media does not allow high and reproducible levels of transfection.

Many known compounds used for transfection experiments are toxic for eukaryotic cells. In addition problems may occur in scaling up where the conditioned medium reduces the effect of known transfecting agents.

The present invention is directed to novel compounds which avoid disadvantages associated with known transfection agents.

In one aspect, the invention is concerned with novel compounds which are conjugates of lipids and a modified basic membrane disturbing peptide, characterized in that the peptides comprise a reversed amide backbone.

The term "conjugates" means compounds consisting of a lipid chemically bound to the peptide, e.g., via a disulfide bond formed between a sulfur atom present in or attached to the lipid

and a sulfur atom present in or attached to the peptide; or an amide bond formed between the carboxyl group present in or attached to the lipid and an amino group of the peptide.

The term "lipid" as used herein comprises straight-chain, branched-chain, saturated or unsaturated aliphatic carboxylic acids and phospholipids. Examples of aliphatic carboxylic acids are lauric acid, palmitic acid, stearic acid, oleic acid and $(CH_3(CH_2)_n)_2CH\ COOH$, where n is an integer from 3 to 19. Examples of phospholipids are phosphatidylethanolamines such as dioleoylphosphatidylethanolamine.

The term "basic peptides" denotes peptides containing at least one basic amino acid. Examples of such basic amino acids are natural and unnatural diamino-monocarboxylic acids, such as alpha-, beta-diaminopropionic acid, alpha-, gamma-diaminobutyric acid, lysine, arginine, ornithine and p-aminophenylalanine, etc.

The term "membrane disturbing peptides" denotes cell-lytic or antibacterial peptides that perturb the barrier function of membranes (G. Saberwal and R. Nagaraj, BBA, 1994, 1197, 109-131). Examples of basic, cell-lytic peptides are melittin, hemolysin, mastoparan, bombolitin, crabrolin and derivatives thereof. Examples of basic antibacterial peptides are cecropins, magainins, gramicidin S and tyrocidine and derivatives thereof.

The term "derivatives" refers to peptides wherein one or more amino acid residues are missing, have been added or have been replaced by another amino acid residue without substantially changing the biological activity of the original peptide concerned, i.e. allow transfection of a macromolecule, preferably a polynucleotide, into a cell. The term "derivatives" also refers to peptides wherein the terminal carboxyl group is esterified, particularly to form lower alkyl esters such as the methyl and ethyl ester; or converted into an amide, lower alkyl amide or di-lower alkyl amide or hydrazide. The term "derivatives" also relates to peptides wherein the NH_2-group of the N-terminus may be acylated to form an amide or a lower alkyl amide. In particular, the NH_2-group is acetylated. The term "lower" denotes groups containing from 1-6 carbon atoms.

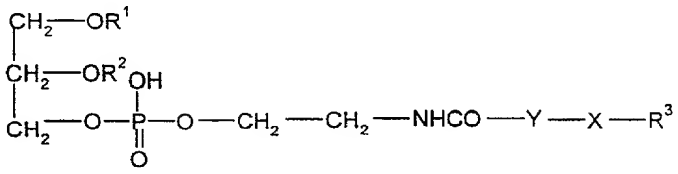
The term "reversed amide backbone" refers to retro-peptides which characteristically have the same composition as its parent peptide, but the sequence is reversed, i.e., n, ... 3, 2, 1 instead of 1, 2, 3, ... n when reading both in the N to C direction. Both have normal peptide bonds.

Throughout this application the following standard abbreviations are used to refer to amino acids:

Alanine	Ala	A
Glutamate	Glu	E
Glutamine	Gln	Q
Aspartate	Asp	D
Asparagine	Asn	N
Leucine	Leu	L
Glycine	Gly	G
Lysine	Lys	K
Serine	Ser	S
Valine	Val	V
Arginine	Arg	R
Threonine	Thr	T
Proline	Pro	P
Isoleucine	Ile	I
Methionine	Met	M
Phenylalanine	Phe	F
Tyrosine	Tyr	Y
Cysteine	Cys	C
Tryptophan	Trp	W
Histidine	His	H
Ornithine	Orn	O
D-Alanine	D-Ala	a
D-Glutamate	D-Glu	e
D-Glutamine	D-Gln	q
D-Aspartate	D-Asp	d
D-Asparagine	D-Asn	n
D-Leucine	D-Leu	l
D-Lysine	D-Lys	k
D-Serine	D-Ser	s
D-Valine	D-Val	v
D-Arginine	D-Arg	r
D-Threonine	D-Thr	t
D-Proline	D-Pro	p

D-Isoleucine	D-Ile	i
D-Methionine	D-Met	m
D-Phenylalanine	D-Phe	f
D-Tyrosine	D-Tyr	y
D-Cysteine	D-Cys	c
D-Tryptophan	D-Trp	w
D-Histidine	D-His	h
D-Ornithine	D-Orn	o

In a preferred embodiment, the novel compounds of the present invention are compounds of formula



(I)

wherein R^1 and R^2 are a hydrocarbyl moiety of a straight-chain or branched-chain, saturated or unsaturated aliphatic carboxylic acid or a phospholipid moiety, R^3 is a basic, membran-edisturbing peptide with a reversed amide backbone, Y is C_{2-10} alkylene, X is -C(O)-NH- or -S-S-, and salts thereof.

Particularly preferred compounds are those wherein R^1 and R^2 independently are an acyl moiety of a C_{12-20} carboxylic acid. The term "C_{12-20}" denotes a number of carbon atoms of from 12 to 20. The acyl moieties R^1 and R^2 can be a straight-chain or branched-chain, saturated or unsaturated moiety. Examples of such moieties are lauroyl, palmitoyl, stearoyl and oleoyl. In a preferred aspect, R^1 and R^2 are oleoyl. Y is preferably ethylene, propylene or decamethylene. X is preferably -S-S-.

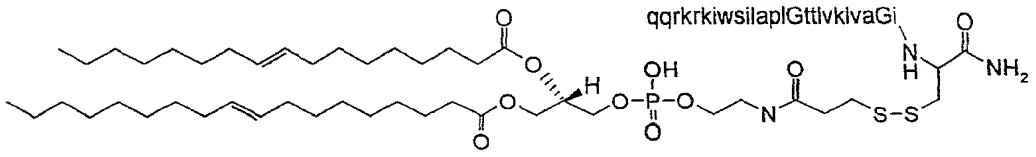
R^3 is a basic, membrane disturbing peptide with a reversed amide backbone. For example, R^3 is Gln-Gln-Arg-Lys-Arg-Lys-Ile-Trp-Ser-Ile-Leu-Ala-Pro-Leu-Gly-Thr-Thr-Leu-Val-Lys-Leu-Val-Ala-Gly-Ile-NH-CH[$CONH_2$]-(CH_2)-. The peptide has a reversed amide backbone. Preferably, R^3 comprises at least 50%, more preferably 65%, and even more preferably 80% of D-amino acids or derivatives thereof. In the most preferred embodiment all amino acids are D-amino acids. The term "D-amino acid" refers to naturally as well as non-naturally D-α-amino carbonic acids or derivatives thereof. In addition R^3 also comprises membrane-disturbing peptide sequences such as magainin, cecropin, defensins, etc., or chimers of such e.g.,

cecropin-melittin (Hancock & Lehrer TIBTECH, 1998, vol. 16, 82-88) synthesized as the retro-inverso peptides and derivatized to allow conjugation to lipids, analogous to the methods as described below, especially analogous in fashion to Example 1.

The term "retro-inverso peptide" is the all –D analogue of an all –L retro peptide.

In a further preferred embodiment, R^3 is D-Gln-D-Gln-D-Arg-D-Lys-D-Arg-D-Lys-D-Ile-D-Trp-D-Ser-D-Ile-D-Leu-D-Ala-D-Pro-D-Leu-Gly-D-Thr-D-Thr-D-Leu-D-Val-D-Lys-D-Leu-D-Val-D-Ala-Gly-D-Ile-NH-[$CONH_2$]-CH-(CH_2)-.

The most preferred compound is



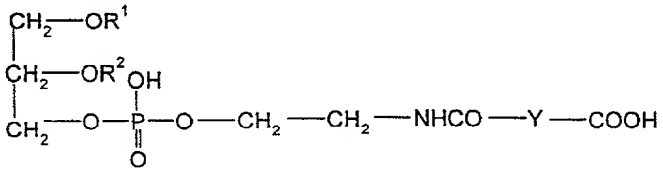
A further embodiment of the present invention refers to the peptide portion of R^3, especially to the intermediate peptide Gln-Gln-Arg-Lys-Arg-Lys-Ile-Trp-Ser-Ile-Leu-Ala-Pro-Leu-Gly-Thr-Thr-Leu-Val-Lys-Leu-Val-Ala-Gly-Ile-Cys-NH_2 or derivatives and/or salts and/or solvates thereof having a reversed amide backbone and consisting of at least 50%, more preferably 65%, and even more preferably 80% of D-amino acids or derivatives thereof. In the most preferred embodiment all amino acids are D-amino acids. The term "D-amino acid" refers to naturally as well as non-naturally D-α-amino carbonic acids or derivatives thereof.

In a preferred embodiment the peptide is D-Gln-D-Gln-D-Arg-D-Lys-D-Arg-D-Lys-D-Ile-D-Trp-D-Ser-D-Ile-D-Leu-D-Ala-D-Pro-D-Leu-Gly-D-Thr-D-Thr-D-Leu-D-Val-D-Lys-D-Leu-D-Val-D-Ala-Gly-D-Ile-D-Cys-NH_2.

In another aspect, this invention relates to a process for preparing the novel compounds defined above, i.e., conjugates of lipids and basic, membrane disturbing peptides wherein the peptides comprise a reversed amide backbone, and compositions comprising at least one such compound, a polynucleotide or any other anionic macromolecule, and, optionally, a helper lipid and/or a short chain phospholipid, and/or a cationic lipid and optionally an additional known transfection reagent other than a conjugate of this invention (i.e. a compound of formula I or II). In still another aspect, this invention relates to compositions comprising conjugates of lipids and basic, membrane disturbing peptides and a helper lipid and/or a short

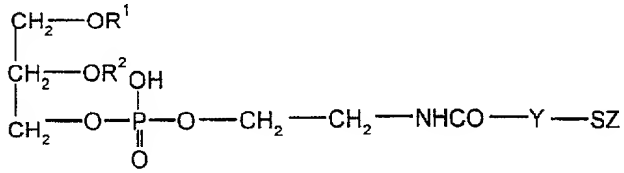
chain phospholipid, and/or a cationic lipid or and optionally an additional known transfection reagent other than a conjugate of this invention, e.g. a compound of formula I. The invention further relates to the use of the novel compounds as a carrier for transfecting a cell with a polynucleotide or any other anionic macromolecule.

The compounds provided by this invention can be prepared by reacting a peptide of the formula R^3NH_2 with a lipid of the formula



II

or a peptide of the formula R^3SH with a lipid of the formula



III

wherein R^1, R^2, R^3 and Y are as defined above and Z is a leaving group such as 2-pyridinethio. These reactions can be carried out in a manner known per se.

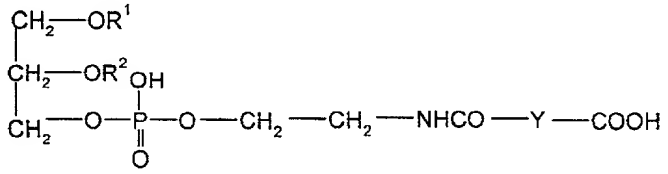
Compounds of the formula R^3NH_2 wherein the peptide part is comprises a reversed amide backbone may be prepared by methods known in the art. The corresponding methods have been described for example for the preparation of synthetic melittin, its enantio, retro, and retroenantio isomers and derivatives thereof (Juvvadi et al. (1996) J. Am. Chem. Soc. 118, 8989-8997).

The peptides may be prepared by the solid-phase synthesis technique. In this technique, synthesis is occurring while the peptide is attached to a polymeric support, therefore allowing for the separation of product from byproduct by washing steps. The completion of the acylation reaction is ensured by using large excesses of soluble reagents. Synthesis involves the covalent anchorage of the first amino acid in the sequence to the solid support followed by the deprotection of the protected amino function for the subsequent coupling to the incoming amino acid derivative. After n cycles of deprotection and coupling, the peptide is released from the solid phase by a chemical cleavage reaction. In a preferred embodiment, D-amino acids are used.

Thus, the coupling of peptide of the formula R^3NH_2 with a lipid of the formula II can be accomplished by reacting the compounds wherein amino group other than the amino group to be reacted are protected in a suitable solvent in the presence of a condensation agent such as dicyclohexylcarbodiimide in analogy to methods known for producing peptide bonds.

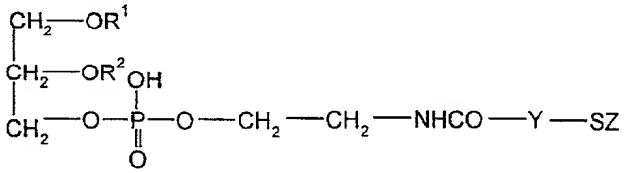
The reaction of a peptide of the formula R^3SH with a compound of formula R-SZ can be carried out in an appropriate solvent or solvent mixture which solubilizes both reactants. The compound of formula R-SZ can be dissolved in an organic solvent, e.g., in chloroform. The peptide R^3SH is suitably dissolved in aqueous buffer solution, such as phosphate buffer, that contains an appropriate amount of an water-miscible organic solvent such as acetonitrile to accomplish the formation of a single phase reaction system.

Compounds of the formula II and III are known or can be prepared by known methods, e.g. as described in Biochim. Biophys. Acta, 1986 862, 435-439. For example, compounds of the formula



II

wherein R^1 and R^2 are oleolyl, and Y is ethylene, propylene or decamethylene and the compound of the formula



III

wherein R^1 and R^2 are oleolyl, Y is ethylene and Z is 2-pyridinethio are commercially available as N-Succinyl-PE, N-Glutaryl-PE , N-Dodecanyl-PE and N-PDP-PE from Avanti Polar Lipids, Alabaster, Alabama, USA.

Any anionic macromolecule can be transfected into a cell using a compound of formula I. An anionic macromolecule is a macromolecule which contains at least one negative charge per molecule. Examples of anionic macromolecules which can be transfected in accordance with this invention include polynucleotides , such as deoxyribonucleic acids (DNA) and ribo-

nucleic acids (RNA); and proteins, such as ribonucleoproteins and proteins used for immunization, e.g. viral proteins. Examples of DNA for use in the present invention are plasmids and genes, especially those for which gene therapy protocols have been launched such as cystic fibrosis transmembrane regulator (CFTR), adenosine deaminase (ADA), thymidine kinase (tk) and HLA B7; as well as reporter genes such as beta-galactosidase, luciferase, green fluorescence protein (gfp), chloramphenicol transferase and alpha-1 antitrypsin. Other examples of DNA are oligodeoxynucleotides and their analogues used as antisense, aptamer or 'triple-helix' agents. Examples of RNA are ribozymes or oligoribonucleotide antisense molecules.

The nature of the cell which is to be transfected is not narrowly crucial. The cell can be a prokaryotic or eukaryotic cell, a mammalian or a plant cell.

In transfecting a cell using a conjugate of this invention, e.g. a compound of formula I, the cell is contacted with the anionic macromolecule in the presence of an appropriate amount of such compound. The appropriate amount of the conjugate, e.g. a compound of formula I for a given amount of anionic macromolecule depends on their respective charges. The +/- charge ratio between the conjugate and the molecule to be transfected generally varies between 0.1-10, preferably between 0.5-5. The value of "+/- charge ratio" is calculated by dividing the number of positively charged groups on the amino acids in the group R^3 by the number of negative charges of the molecule to be transfected. When the molecule to be transfected is a polynucleotide for example, number of negative charges means the number of negatively charged phosphates in the backbone. The optimal ratio within these ranges depends on the cell to be transfected and is readily ascertained by one of skill in the art to which this invention pertains.

The amount of anionic macromolecules to the number of cells is such that the amount of anionic macromolecule for transfecting 10^4 cells is from 0.1 ng to 10 mg, preferably from 0.2 mg to 2 mg. When the anionic macromolecule is DNA the preferred amount of DNA for transfecting 10^4 cells in vitro is from 0.1 mg to 10 mg. When cells are being transfected in vivo, the preferred amount of DNA is from 0.1 μg to 1 g.

In a preferred aspect of the invention the transfection is further carried out in the presence of a helper lipid and/or short chain phospholipid, and/or a cationic lipid or any other known transfection competent molecule other than a conjugate of this invention. Any conventional helper lipid can be used. "Helper lipids" are phospholipids which are known to increase delivery of macromolecules to cells when used together with known transfection competent molecules. Examples of helper lipids are phospholipids, such as phosphatidylcholines or phosphatidyl-

ethanolamines or mixtures thereof. Preferred helper lipids are phosphatidylethanolamines, such as dioleoylphosphatidylethanolamine. Any conventional short chain phospholipid can be used. "Short chain phospholipids" are phospholipids containing fatty acid residues, which fatty acid residues contain from 6 to 12 carbon atoms in their backbone. Examples of short chain phospholipids are phosphatidylcholines that carry two C_{6-12} fatty acid residues. Preferred short chain phospholipids are dicapryl- and dicapryloyl phosphatidylcholine.

Examples of transfection competent molecules include cationic lipids as described by J.B. Behr in Bioconjugate Chem., 1994, 5,382-389 and X. Gao and L. Huang in Gene Ther., 1995, 2, 710-722; polycations as described by A.V. Kabanov and V.A.: Kabanov in Bioconjugate Chem., 1995, 6, 7-20; peptides and polymers and other non-viral gene delivery systems as described by F.D. Ledley in Human Gene Therapy, 1995, 6, 1129-1144.

The helper lipid and/or short chain phospholipid and/or a cationic lipid and optionally another additional known transfection competent molecule other than a conjugate of this invention is suitably in the form of a liposome, micelles, organic or aqueous dispersions, or organic or aqueous solutions. The optimal molar ratio between the compound of formula I and the helper lipid is 0.1:50, preferably 1:10. The optimal molar ratio between helper lipid and short-chain phospholipid is 2:20. The optimal molar ratio between the compound of formula I or II and additional transfection competent molecules is 0.1:10.

The present invention also comprises the use of a composition as defined above for transfecting an eukaryotic or prokaryotic cell in vivo or in vitro with an anionic macromolecule, preferably with a polynucleotide.

The invention also comprises the use of compounds of formula (I) as defined above for transfecting a eukaryotic or prokaryotic cell in vivo or in vitro with an anionic macromolecule, preferably with a polynucleotide.

A further embodiment of the present invention is a process for transfecting a cell in vivo or in vitro with an anionic macromolecule, preferably a polynucleotide, comprising contacting a cell in vivo or in vitro with the anionic macromolecule in the presence of a compound of formula (I) or with a composition as defined above.

In addition, the invention is directed to a process for introducing a biologically active anionic molecule into a cell in vivo or in vitro with an anionic macromolecule, comprising contacting a cell in vivo or in vitro with the anionic macromolecule in the presence of a composition or a compound as defined above. This includes also a process for introducing in vivo or in vitro a

biologically active anionic molecule into a cell, comprising contacting in vivo or in vitro a cell with the anionic macromolecule in the presence of a composition or compound as defined above.

Further, the present invention is directed to the use of a compound or composition as defined above for introducing in vivo or in vitro a biologically active molecule into cells.

For transfection, an appropriate amount of a conjugate of this invention, e.g., a compound of formula I is added to the molecule to be transfected (e.g., plasmid DNA), suitably in an aqueous solution. Optionally, a helper lipid and, if desired, a short chain phospholipid and/or a cationic lipid and optionally another additional known transfection competent molecule other than a conjugate of this invention is then added, either in form of liposomes, micelles, or as an organic solution or aqueous dispersion. Alternatively, the molecule to be transfected may be added to a composition comprising a compound in accordance with this invention, a helper lipid, and, if desired, a short chain phospholipid and/or a cationic lipid and optionally another additional known transfection competent molecule other than a conjugate of this invention. The composition may be in solid, liquid, semisolid or aerosol form, suitably in form of liposomes, micelles, or as an organic solution or aqueous dispersion.

For transfecting cells in an animal or human patient the composition can be administered by oral, parenteral (i.v., i.m., s.c., i.d., i.p.) transdermal, pulmonary, nasal, rectal, ocular, ventricular, vascular (catheter) and intratumoral route. Furthermore, the composition can be administered by high velocity impaction administration to the skin surface. The progress of transfection can be measured by appropriate testing protocols which are known to those skilled in the art.

The present invention also refers to a composition comprising at least one compound as defined above and a helper lipid and/or a short chain phospholipid and/or a cationic lipid or optionally another additional transfection competent molecule. In addition, the composition may comprise an anionic macromolecule, preferably a polynucleotide. These compositions may also comprise a polycationic polymer, preferably polyethyleneimine (PEI). The components of these compositions may be in the form of an aqueous or organic solution, an aqueous or organic dispersion, or a liposome or a micelle.

The term "polyethylenimine (PEI)" refers to a synthetic organic, generally branched macromolecule with a high cationic charge/density potential, preferably with a molecular weight of about 25 kDA.

The invention also relates to the use of a compound as above for transfecting a cell with an anionic macromolecule, preferably a polynucleotide.

In accordance with the present invention it has been found that conjugating a lipid to a basic, membrane disturbing peptide having a reversed amide bond results in novel compounds that bind polynucleotides and other anionic macromolecules and can be used for transfection of cells. Thus, the invention is concerned with a process for transfecting a cell with an anionic macromolecule comprising, contacting the cell with the anionic macromolecule in the presence of a compound as described above, so as to transfect the cell with the anionic macromolecule.

The invention also relates to a process for production of large quantities of recombinant proteins in PEI-mediated transfected cells. For example, high-level expression of both G protein coupled receptors an ligand-gated ion channels by the use of Semliki Forest virus (SFV) has been realized. Generally, B_{max} values of more than 50 pmol receptor per mg protein and receptor densities of more than 3×10^6 receptors per cell have been achieved. To further facilitate large scale protein production optimal conditions for mammalian serum-free suspension cultures have been obtained. Adaptation of BHK, CHO and HEK293 cells to these conditions has allowed efficient infection with SFV vectors to produce large volumes of recombinant protein expressing cell cultures.

Surprisingly, the growth temperature of the cell cultures can have a dramatic effect on the duration and levels of recombinant protein-expression. Expression of recombinant luciferase is increased 5 to 10-fold by lowering the growth temperature of BHK and CHO cells from 37°C to 33°C. The expression time is much longer at 33°C with still high expression 65 hours post-infection.

Moreover, the effect of the temperature on the expression of two 7TM receptors, human neurokinin-1 receptor and rat metabotropic glutamate receptor-2 and the 5-HT_3 ligend-gated ion channel could be shown. A similar effect as observed for luciferase has also been obtained for the receptors. The receptor density is much higher in cells grown at 33°C compared to 37°C. The expression time for receptors is usually restricted to 24 hours in SFV-infected cells, but can be prolonged to 65 hours when cells are cultured at 33°C. This improvement can greatly facilitate production of large quantities of recombinant proteins. Moreover, the utility of the inventive transfection for the rapid expression of heterologous proteins in large scale for adherent cells (CFU) and suspension cells (12 l, 23 l, 60 l fermentors) has been realized. In particular helper lipids in combination with PEI exhibit a high transient gene expression in HEK293 cells and other mammalian cell lines at low DNA concentration. In addition the fre-

quently occurring inhibitory effect of conditioned medium which is a serious problem for scale up expression methods is reduced or even avoided.

The following examples which are not limitative illustrate the invention further.

EXAMPLES

Example 1

a) Preparation of the retro-inverso peptide R^3SH

Continuous-flow solid-phase synthesis was performed on a Pioneer™ Peptide Synthesis System, starting from Tenta Gel S RAM resin (0.25 mmole/g [Rapp Polymere GmbH, Tübingen, Germany] according to the method described by Atherton and Sheppard, Solid Phase Peptide Synthesis : A Practical Approach (IRL Press Oxford 1989). The base-labile Fmoc group was used for a-amino protection. Side chains were protected with the following protection groups: D-Arg(Pbf),D-Cys(Trt),D-Gln(Trt),D- Lys(Boc),D-Ser(But), D-Thr(But) and D-Trp(Boc). Fmoc-amino acids (2.5 equiv.) were activated with an equivalent amount of TATU (L. A. Carpino J. Am. Chem. Soc. 1993, 115, 4397-4398) and DIPEA. Fmoc deprotection was achieved with 20% piperidine in DMF. D-Gln(Trt)- D-Gln(Trt)- D-Arg(Pbf)- D-Lys(Boc)- D-Arg(Pbf)- D- Lys(Boc)-D-Ile- D-Trp(Boc)- D-Ser(But)-D-Ile-D-Leu-D-Ala-D-Pro-D-Leu-Gly-D-Thr(But)- D-Thr(But)-D-Leu-D-Val- D- Lys(Boc)-D-Leu-DVal-D-Ala-Gly-D-Ile-D-Cys(Trt) -amide Tenta Gel S-resin (2.0 g) was treated with a mixture (100 ml) of 90% TFA, 2% EDT, 5% H_2O, 3% triisopropylsilane for 6 hours. The reaction mixture was concentrated and poured into diethyl ether and the precipitate was collected by filtration and lyophilized from water. The crude peptide was purified by preparative RP-HPLC. There was obtained homogenous D-Gln-D-Gln-D-Arg-D-Lys-D-Arg-D-Lys-D-Ile-D-Trp-D-Ser-D-Ile-D-Leu-D-Ala-D-Pro-D-Leu-Gly-D-Thr-D-Thr-D-Leu-D-Val-D-Lys-D-Leu-D-Val-D-Ala-Gly-D-Ile-D-Cys-NH_2. 6 TFA.

b) Preparation of a compound of formula IV

The homogenous peptide obtained in paragraph a) above (24.2 mg, 7 mmole) was dissolved in a mixture of 2 ml of 100 mM ammonium acetate buffer, pH 6.5, and 2 ml of acetonitrile. To this solution there was added 7.4 mg of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[3-(2-pyridyldithio)propionate] in 0.5 ml of chloroform. The mixture was stirred at room temperature for 1 hour and the organic solvent was removed by evaporation. The remaining solution was washed 3 times with ethyl acetate and the aqueous phase was lyophilized. There was obtained 28.5 mg of a compound of formula IV wherein R^1 and R^2 are oleoyl and D-Gln-D-Gln-D-Arg-D-Lys-D-Arg-D-Lys-D-Ile-D-Trp-D-Ser-D-Ile-D-Leu-D-Ala-D-Pro-D-Leu-Gly-D-Thr-D-Thr-D-Leu-D-Val-D-Lys-D-Leu-DVal-D-Ala-Gly-D-Ile-NH-CH[$CONH_2$]-(CH_2)- is R^3. ISP-MS: M= 3722.

Example 2

1 mg of the compound obtained in Example 1 was dissolved in 500 µl acetonitrile and diluted in 500 µl sterile pure water (1mg/ml) and stored at 4°C. 15 µl of a plasmid solution (1mg/ml) and stored at 4°C. 15 µl of a plasmid solution encoding the soluble human tumor necrosis factor receptor p55 gene was transferred into 1.5 ml medium and mixed. Various amounts of the compound obtained in a) was added , mixed and after 10 min at room temperature the mixture was transferred to 15 ml HEK293(EBNA) cells in suspension.

Table 1 shows the transfection efficiency of the compound as described in Example 1 and the cell viability in serum-free suspension culture.

Table 1

Transient tranfection of HEK293(EBNA) cells					
Compound (mg/ml)	1	2	4	6	10
viability (%)	90 – 95	90 – 95	90 – 95	90 – 95	90 – 95
TNFRp55 (ng/ml)	0.1	0.2	2.5	6.3	83

The data clearly indicate that compound-mediated transfection can be achieved in the absence of detectable cell toxicity effects in serum-free suspension culture of 293 (EBNA) cells.

Example 3

1 mg of the compound obtained in Example 1 was dissolved in 500 µl acetonitrile and diluted in 500 µl sterile pure water (1mg/ml) and stored at 4°C. 90 mg polyethyleneimine (PEI), with a molecular weight of 25 kDa, were dissolved in sterile pure water (0.9 mg/ml), neutralized with HCl and sterile filtered and stored at room temperature.

3 µl of a plasmid solution (1mg/ml) encoding the soluble human tumor necrosis factor receptor p55 gene was diluted in 1.5 ml culture medium and mixed. Various amounts of the compound (1mg/ml) as described in Example 1 were added, mixed, followed by 8.3 µl PEI

(0.9 mg/ml). After mixing and incubation for 10 min at room temperature the mixture was transferred into 15 ml of HEK293 (EBNA) cells in suspension. Cells which were grown in serum-free suspension culture were transferred into fresh medium before adding the complexes. The released receptor protein was measured 72 hrs post-transfection in the culture medium and expressed as ng receptor per milliliter culture.

Table 2 shows the transfection efficiency of the compound described in Example 1 in combination with PEI by measuring the released receptor protein in serum free suspension culture.

Table 2

Transient transfection of HEK293(EBNA) cells together with PEI					
Compound + polyethyleneimine (mg/ml)	0.02 + 0.5	0.05 + 0.5	0.1 + 0.5	0.15 + 0.5	0 + 0.5
TNFRp55 (ng/ml)	490	800	1120	1100	180

The results clearly indicate the synergisitc enhancement of the coumpound together with PEI. The sequence of adding first the compound followed by PEI to the DNA or vice versa has no detectable infludence on the transfection effeciency. No significant changes of the expression levels were observed when the cells were cultured in the presence of 10% serum.

Example 4

1 mg of the compound obtained in Example 1 was dissolved in 500 µl acetonitrile and diluted in 500 µl sterile pure water (1mg/ml) and stored at 4°C. 90 mg polyethelenimine, with a molecular weight of 25 kDa, was dissolved in sterile pure water (0.9 mg/ml), neutralized with HCl and sterile filtered and stored at room temperature.

3 µl of a plasmid solution (1 mg/ml) encoding the luciferase gene was diluted in 1.5 ml culture medium and mixed. 2.25 µl compound (1mg/ml) as described in Example 1 was added, mixed, and followed by 8.3 µl PEI (0.9 mg/ml). After mixing and incubation for 10 min at room temperature the mixture was transferred into 15 ml of various cells. Cells which were

grown in serum-free suspension culture were transferred into fresh medium before adding the complexes. The luciferase activity was measured the soluble cell extract after 24 hrs incubation and expressed as relative light unit (RLU) per milligram protein.

Table 3 shows the transfection efficiency of the compound described in Example 1 in combination with PEI by measuring the luciferase activity in serum free suspension culture.

Table 3

	Luciferase activity (RLU/ml)		
	Compound	**PEI**	**Compound + PEI**
BHK 21 C1	5.2×10^5	2.8×10^7	1.8×10^9
CHO dhfr-	1.4×10^6	3.5×10^7	5.3×10^9
HEK293 (EBNA)	2.2×10^6	3.2×10^9	6.7×10^{10}
HEK293	1.8×10^6	2.9×10^9	6.3×10^{10}
Glioma C6	5.8×10^6	6.5×10^7	3.4×10^9

The results clearly indicate the synergistic enhancement of the compound together with PEI in various cell lines.

Example 5

1 mg of the compound obtained in Example 1 was dissolved in 50 µl acetonitrile and diluted in 500 µl sterile pure water (1mg/ml) and stored at 4°C. 90 mg polyethelenimine, with a molecular weight of 25 kDa, was dissolved in sterile pure water (0.9 mg/ml), neutralized with HCl and sterile filtered and stored at room temperature.

3 ml of a plasmid solution (1mg/ml) encoding the green fluorescence protein was diluted in 1.5 ml culture medium and mixed. 2.25 µl compound (1mg/ml) was added, mixed, and followed by 8.3 µl PEI (0.9 mg/ml). After mixing and incubation for 10 min at room temperature the mixture was transferred into 15 ml of various cells. Cells which were grown in serum-free suspension culture were transferred into fresh medium before adding the com-

plexes. The transfection efficiency was measured by counting the number of fluorescent cells after 24 hours incubation and expressed as percent transfected cells.

Table 4 shows the transfection efficiency of the compound described in Example 1 in combination with PEI by measuring the green fluorescence within various cell lines in serum-free suspension culture.

Table 4

Amount of fluorescence cells (%) - compound + PEI	
BHK 21 C1	25 (± 5)
CHO dhfr$^-$	15 (± 5)
HEK293(EBNA)	85 (± 5)

Example 6

1 mg of the compound obtained in Example 1 was dissolved in 500 µl acetonitrile and diluted in 500 µl sterile pure water (1 mg/ml) and stored at 4°C. 90 mg polyethylenimine, with a molecular weight of 25 kDa, was dissolved in sterile pure water (0.9 mg/ml), neutralized with HCl and sterile filtered and stored at room temperature.

3 ml of a plasmid solution (1 mg/ml) encoding the luciferase gene was diluted in 1.5 ml culture medium and mixed. 2.25 µl compound (1 mg/ml) as described in Example 1 was added, mixed, and followed by 8.3 µl PEI (0.9 mg/ml). After mixing and incubation for 10 min at room temperature 0.1 ml of the mixture was transferred into a well containing 1 ml medium of a 12 well plate with various adherent cell lines. Cells which were grown in the presence of 10% serum were incubated during 4 hours post-transfection in serum-free medium. The luciferase activity was measured in the soluble cell extract after 24 hours incubation and expressed as relative light unit (RLU) per milligram protein.

Table 5 shows the transfection efficiency of the compound described in Example 1 in combination with PEI by measuring the luciferase activity in various adherent cell lines.

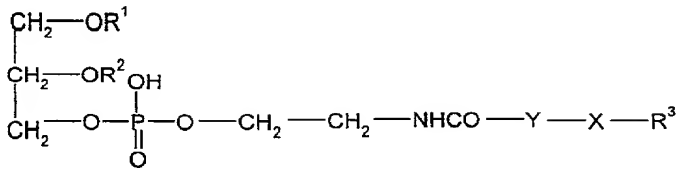
Table 5

	Luciferase activity (RLU/mg Protein)	
	PEI	**Compound PEI**
HEK293	5.4×10^9	5.3×10^{10}
glioma C6	3.1×10^9	1.9×10^{10}
IMR32	2.2×10^9	7.7×10^9
COS 7	2.9×10^8	1.1×10^{10}
HepG2	$<1 \times 10^8$	8×10^8

The results clearly indicate also the synergistic enhancement of the compound together with PEI in various adherent cell lines.

CLAIMS

1. Conjugates of lipids and basic, membrane disturbing peptides which are compounds of formula



(I)

wherein R^1 and R^2 are a hydrocarbyl moiety of a straight-chain or branched-chain, saturated or unsaturated aliphatic carboxylic acid or a phospholipid moiety, R^3 is a basic, membrane disturbing peptide with a reversed amide backbone, Y is C_{2-10} alkylene, X is -C(O)-NH- or -S-S- and salts thereof.

2. The compounds of claim 1 wherein R^1 and R^2 independently are an acyl moiety of a C_{12-20} carboxylic acid.

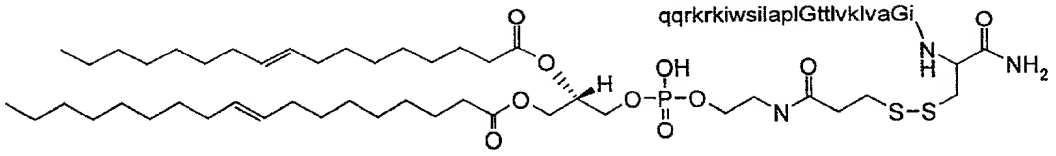
3. The compounds of claims 1 or 2 wherein R^1 and R^2 are independently selected from lauroyl, palmitoyl, stearoyl and oleoyl.

4. The compounds of claims 1 - 3 wherein X is -S-S-.

5. The compounds of claims 1 - 4 wherein R^3 is Gln-Gln-Arg-Lys-Arg-Lys-Ile-Trp-Ser-Ile-Leu-Ala-Pro-Leu-Gly-Thr-Thr-Leu-Val-Lys-Leu-Val-Ala-Gly-Ile-NH-CH[$CONH_2$]-(CH_2)- with a reversed amide backbone or derivatives thereof consisting of at least 50 % D-amino acids.

6. The compounds of claim 1 - 5 wherein R^3 is D-Gln-D-Gln-D-Arg-D-Lys-D-Arg-D-Lys-D-Ile-D-Trp-D-Ser-D-Ile-D-Leu-D-Ala-D-Pro-D-Leu-Gly-D-Thr-D-Thr-D-Leu-D-Val-D-Lys-D-Leu-D-Val-D-Ala-Gly-D-Ile-NH-[$CONH_2$]-CH-(CH_2)-.

7. The compound according to any of claims 1 - 6, which is



(IV)

8. The peptide Gln-Gln-Arg-Lys-Arg-Lys-Ile-Trp-Ser-Ile-Leu-Ala-Pro-Leu-Gly-Thr-Thr-Leu-Val-Lys-Leu-Val-Ala-Gly-Ile-Cys-NH_2 with a reversed amide backbone and consisting of at least 50 % D-amino acids or derivatives.

9. The peptide of claim 8 which is D-Gln-D-Gln-D-Arg-D-Lys-D-Arg-D-Lys-D-Ile-D-Trp-D-Ser-D-Ile-D-Leu-D-Ala-D-Pro-D-Leu-Gly-D-Thr-D-Thr-D-Leu-D-Val-D-Lys-D-Leu-D-Val-D-Ala-Gly-D-Ile-D-Cys-NH_2 and/or salts and/or solvates thereof.

10. A composition comprising at least one compound as defined in any one of claims 1-7 and a helper lipid and/or a short chain phospholipid and/or a cationic lipid and optionally an additional transfection reagent.

11. The composition of claim 10 comprising in addition an anionic macromolecule, preferably a polynucleotide.

12. The composition of claims 10 or 11 comprising in addition a polycationic polymer, preferably polyethyleneimine.

13. The composition of claims 10 - 12, wherein the components are in the form of an aqueous or organic solution, an aqueous or organic dispersion, or a liposome or a micelle.

14. Use of a composition as defined in any one of claims 10 - 13 for transfecting a eukaryotic or prokaryotic cell in vivo or in vitro with an anionic macromolecule.

15. Use of a composition as defined in any one of claims 10 - 13 for transfecting a eukaryotic or prokaryotic cell in vivo or in vitro with a polynucleotide.

17. Use of a compound as defined in any one of claims 1 - 9 for transfecting a cell in vivo or in vitro with a polynucleotide.

18. A process for transfecting a cell in vivo or in vitro with an anionic macromolecule, comprising contacting a cell in vivo or in vitro with the anionic macromolecule in the presence of a compound as defined in any of claims 1 - 9.

19. A process for transfecting a cell in vivo or in vitro with an anionic macromolecule, comprising contacting a cell in vivo or in vitro with the anionic macromolecule in the presence of a composition as defined in any of claims 10 -13.

20. Use of a compound as defined in any one of claims 1 - 9 for introducing in vivo or in vitro a biologically active molecule into cells.

21. Use of a composition as defined in any one of claims 10 - 13 for introducing in vivo or in vitro a biologically active molecule into cells.

22. A process for introducing a biologically active anionic molecule into a cell in vivo or in vitro with an anionic macromolecule, comprising contacting a cell in vivo or in vitro with the anionic macromolecule in the presence of a composition as defined in any of claims 10 - 13.

23. A process for introducing in vivo or in vitro a biologically active anionic molecule into a cell, comprising contacting a cell in vivo or in vitro with the anionic macromolecule in the presence of a compound as defined in any of claims 1 - 9.

24. A process for introducing in vivo or in vitro a biologically active anionic molecule into a cell, comprising contacting in vivo or in vitro a cell with the anionic macromolecule in the presence of a composition as defined in any of claims 10 - 13.

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Application

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Application Number	09/647,586
Filing Date	02 October 2000
First Named Inventor	KITAS et al.
Group Art Unit	
Examiner Name	
Attorney Docket Number	RDID 0063 US

Please change the Correspondence Address for the above-identified application to:

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OR

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I am the :

☐ Applicant.

☐ Assignee of record of the entire interest.
Certificate under 37 CFR 3.73(b) is enclosed.

XX ☐ Attorney or agent of record .

Typed or Printed Name	Jill L. Woodburn Reg. 39,874
Signature	Jill L. Woodburn
Date	December 22, 2000

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Assistant Commissioner for Patents
Washington, DC 20231

GENERAL APPOINTMENT OF REPRESENTATIVE FOR U.S. PATENT AND TRADEMARK OFFICE MATTERS

The undersigned applicant or assignee hereby appoints D. Michael Young, Reg. No. 33,819, Richard T. Knauer, Reg. No. 35,575, Brent A. Harris, Reg. No. 39,215, Kenneth J. Waite, Reg. No. 45,189, and Marilyn L. Amick, Reg. No. 30,444 all of Roche Diagnostics Corporation, 9115 Hague Road, P.O. Box 50457, Indianapolis, Indiana 46250, Telephone No. (317) 845-2000, and Jill Lynn Woodburn, Reg. No. 39,874 of The Law Office of Jill L. Woodburn, L.L.C., 6633 Old Stonehouse Drive, Newburgh, Indiana 47630-1785, Telephone No. (812) 842-2660:

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to prosecute and transact all business on its behalf before the United States Patent and Trademark Office in connection with any U.S. patent assigned to it and any U.S. patent application filed by it or on its behalf and to receive payments on its behalf.

Signed this 18th day of September, 2000 at Mannheim, Germany.

Roche Diagnostics GmbH	Roche Diagnostics GmbH
Signature	Signature
Dr. Michael Jung	Dr. Manfred Weber
Print Name	Print Name
Director	Senior Director
Position or Title	Position or Title

Docket No.
RDID0063US

Declaration and Power of Attorney For Patent Application

English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled
NEW COMPOUNDS FOR DNA-TRANSFECTION

the specification of which

(check one)

☐ is attached hereto.

☒ was filed on **October 2, 2000** as United States Application No. or PCT International Application Number **09/647,586**

and was amended on _____
(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Not Claimed
PCT/EP99/02361 (Number)	**PCT** (Country)	**7 April 1999** (Day/Month/Year Filed)	☐
98124837.0 (Number)	**Europe** (Country)	**30 December 1998** (Day/Month/Year Filed)	☐
98106302.7 (Number)	**Europe** (Country)	**7 April 1998** (Day/Month/Year Filed)	☐

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

(Application Serial No.)	(Filing Date)

I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(list name and registration number)*

6

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